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Biallelic *KRT5* mutations in epidermolysis bullosa simplex, including a complete human keratin 5 "knock-out"

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Running title: Biallelic *KRT5* mutations in EBS

Abbreviations: BAM, Binary Alignment Map; CSV, Comma-Separated Values; CVS, Chorionic Villus Sample; DEB, Dystrophic Epidermolysis Bullosa; EB, Epidermolysis Bullosa; EBS, Epidermolysis Bullosa Simplex; KS, Kindler Syndrome; JEB, Junctional Epidermolysis Bullosa; LM-PCR, Ligation-Medicated Polymerase Chain Reaction; MAF, Minor Allelic Frequency; NGS, Next Generation Sequencing; ROH, Regions of Homozygosity; VCF, Variant Call Format; WES, Whole Exome Sequencing;

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Abstract

Epidermolysis bullosa simplex (EBS) is usually inherited as an autosomal dominant disease due to monoallelic gain-of-function mutations in *KRT5* and *KRT14*. Although autosomal recessive forms of EBS have been associated with mutations in at least 10 genes, recessive EBS due to homozygous biallelic *KRT5* mutations has not been reported previously; it has been hypothesized that it would result in prenatal lethality. We sought the genetic causes of EB in a cohort of 512 distinct EB patients by performing whole exome sequencing (WES) and using a disease-targeting next-generation sequencing (NGS) panel of 21 genes. The pathogenicity and consequences of the mutations were determined by expression profiling and at tissue and ultrastructural levels. Two pathogenic, homozygous missense variants of *KRT5* in two patients with generalized EBS and a homozygous null mutation in a patient who died as a neonate from complications of EB were found. The two missense mutations disrupted keratin 5 expression on immunofluorescence microscopy, and the human "knock-out" of *KRT5* showed no RNA and protein expression. Collectively, these findings identify biallelic *KRT5* mutations with a phenotypic spectrum varying from mild, localized and generalized to lethal, expanding the genotypic profile of autosomal recessive EBS.

Keywords: Epidermolysis bullosa simplex; *KRT5* homozygosity; next generation sequencing; phenotype-genotype correlations

Introduction

Epidermolysis bullosa (EB), a heterogeneous group of heritable blistering disorders, is subdivided into four broad categories on the basis of the topographic location of the blistering within the skin. In the simplex type (EBS), the blisters develop within the epidermis, in most cases within the basal keratinocytes; in the junctional forms (JEB), tissue separation occurs within the dermal-epidermal basement membrane, primarily within the lamina lucida; in the dystrophic forms (DEB), tissue separation is below the lamina densa within the upper papillary dermis; and in Kindler syndrome (KS) there are multiple levels of blistering within the skin, even in biopsies from the same patient [1-4]. Sub-classification of the patients into these four broad categories has been helpful in prognostication of the severity and the overall outcome of the disease in an individual patient. However, within each of these four categories, there is tremendous phenotypic variability which, in part, reflects genetic heterogeneity. There are as many as 21 distinct genes which harbor mutations in different families with EB. ~~, and the~~ The types of mutations and their consequences at the mRNA and protein levels, when juxtaposed to environmental factors, primarily external trauma ~~at the extracellular matrix/cell interface~~, determine the overall severity in the phenotypic spectrum of this disease [2, 4-7].

EBS is inherited in most cases in an autosomal dominant pattern, and a large number of dominant-negative mutations in the genes, *KRT5* and *KRT14*, which encode basal keratins 5 and 14, respectively, have been disclosed [8-10]. In addition, autosomal dominant inheritance of EBS has been associated with mutations in the *KLHL24* gene [11-13] and with a distinct *PLEC* missense variant [14]. In autosomal recessive EBS, approximately 10% of cases have been shown to result from mutations in *TGM5* encoding transglutaminase 5. In contrast, relatively few cases harbor mutations in other genes, including *JUP*, *DSP*, *PKP1*, *EXPH5*, *PLEC*, *CD151*, and

DST-e [15]. Some EBS families with the autosomal recessive mode of inheritance have been shown to harbor biallelic loss-of-function mutations in *KRT14*, with the resulting blistering phenotype varying from relatively mild to very severe [16]. Up to now, however, no autosomal recessive families with homozygous biallelic *KRT5* mutations have been reported.

In this study we report three consanguineous EBS families with an autosomal recessive mode of inheritance, with the probands harboring homozygous *KRT5* mutations.

Results

We investigated the molecular genetics of EB patients in a large cohort of consanguineous families by next generation sequencing (NGS) approaches, including the use of a disease-targeted sequencing panel consisting of 21 skin fragility-associated genes as well as by whole exome sequencing (WES) [17-19]. In three consanguineous families, probands were found to have skin blistering and erosions since birth, consistent with EB (Fig. 1). The parents were clinically unaffected, suggesting autosomal recessive inheritance (Fig. 2). All three patients were found to have homozygous *KRT5* mutations.

Case Reports

Family 1: The proband was a 32-year-old female born to first-cousin consanguineous parents of Iranian origin with Kurdish ethnicity (Fig. 2a). There was no family history of genetic diseases. Cutaneous manifestations included trauma-induced blistering and erosions noted at a few days after birth. The blisters were multiple and widespread, and they were particularly severe in the plantar aspects of the feet accompanied with plantar keratoderma (Fig. 1). The blisters were often hemorrhagic and painful and healed with pruritus. There was delayed wound healing due to

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infections. The number, size, and the frequency of blisters were exacerbated by exposure to heat. In addition, nail dystrophy, subungual bullae leading to traumatic onycholysis and hemorrhage followed by nail loss, was reported by the patient. No alopecia was noted or reported.

Mucosal manifestations included frequent ulcerations affecting all areas of oral mucosa including the tongue. She was not able to tolerate the trauma of tooth brushing. The patient reported chronic constipation, painful defecation, and fecal impaction. The proband was also found to have renal disease, with a history of kidney and urinary tract infections.

The patient's weight and height were 49 kg and 160 cm, respectively, with a body mass index of 20.3. Hematologic workup revealed that hemoglobin, red blood cell count and mean corpuscular volume were within the normal limits. Pertinent negative findings included lack of musculoskeletal pathology, tracheo-laryngeal disease, cardiac disease, scarring alopecia, corneal erosions, and cutaneous or other malignancies.

Family 2: The male proband, the second child of a first-cousin consanguineous Syrian family, was first encountered in the clinic at the age of one year (Fig. 2b). Generalized skin blistering since birth and occasional involvement of the oral mucous membranes were reported by the mother. The older brother and both parents did not show evidence of skin fragility. Bullae, up to 25 cm^2 in size, were observed mainly on the soles and the abdomen. Blisters, once pierced with a sterile needle, healed without scarring. The proband, now four years of age, showed regular weight gain but slightly delayed psychomotor development.

Family 3: The female proband was born at 40 weeks' gestation by cesarean section from a Syrian mother with a first-cousin consanguineous marriage (Fig. 2c). Her weight at birth was 2.8 kg. At birth, the patient had extensive skin defects and multiple cutaneous blisters on her feet,

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legs, hands and right arm (Fig. 1). Her legs and feet were somewhat underdeveloped, and the skin was atrophic. Within a few hours after birth, more blisters developed on her trunk, the face, and her mouth, and eventually about 80% of the body was affected. These blisters ruptured easily. Her hands and feet were swollen, and she seemed to experience pain. She was placed in an incubator at the neonatal intensive care unit, an umbilical catheter was inserted, and treatment with antibiotics and analgesics was initiated. The skin heeled on some parts of the body, but not on the feet and hands. One month after birth, she became lethargic, the urine production subsided, her breathing became labored, and she died four weeks after the birth. The mother's sister was born with similar skin manifestations, and she also died shortly after birth.

Whole exome sequencing and NGS panel identified homozygous pathogenic variants in *KRT5*

We have developed a disease-targeted NGS array consisting of 21 genes associated with skin fragility disorders (for the list of genes in the panel, see Materials and Methods). This sequencing panel was applied to DNA from 362 probands, each representing a distinct family, with a clinical diagnosis of EB, based on history of neonatal blistering and erosions of the skin. NGS, followed by bioinformatics analysis, identified in probands of Families 1 and 2 homozygous missense variants, NM_000424.4:c.508G>A, p.Glu170Lys; and NM_000424.4:c.472G>A, p.Asp158Asn in *KRT5*, respectively (Fig. 2a, b). These variants were predicted to be pathogenic by ANNOVAR software tool by using various prediction programs (including Provean, SIFT, PolyPhen-2, Mutation Taster, and Fathmm), and they were not found either in ExAC, 1000 Genomes, or gnomAD databases of normal populations.

The presence of these mutations at the genomic level was confirmed by PCR, followed by bi-directional Sanger sequencing (Fig. 2a,b), and the patients' clinically unaffected parents were shown to be heterozygous carriers of these mutations.

In Family 3, the proband was initially diagnosed with a severe form of EB, based on cutaneous and oral blistering. For confirmation of her diagnosis, the proband's DNA was submitted to WES, which identified a total of 88,210 sequence variants (Fig. 2de). Following bioinformatics filtering, which considered exonic/splicing variants only, removed synonymous and benign sequence variants, and identified homozygous variants with minor allelic frequency (MAF) <0.001, reduced the number of annotated, potentially pathogenic variants to 20 (Fig. 2de; for the complete list, see Table S1).

For elucidation of the degree of homozygosity from the WES data, Binary Alignment Map (BAM) files were converted to Variant Call Format (VCF) format, and VCF files were used as an input for PLINK (see Materials and Methods). The total amount of regions of homozygosity (ROH) identified in the proband was 220 Mb (out of the total autosome of 2,850 Mb) or about 7.8 percent. This percentage is consistent with the parents' reported first cousin relationship and reflects the extensive practice of consanguinity in the extended family. Potentially pathogenic variants overlapping with ROHs in the proband reduced the number of variants to 7 (Fig. 2d), and a recessive homozygous base substitution variant *KRT5*:NM_000424.4:c.1440-1G>A was discovered when the candidate genes were matched with the phenotype. This homozygous *KRT5* mutation resided within a 22.3 Mb ROH in chromosome 12 (Fig. 2e). Thus, this ROH harboring the candidate gene *KRT5* provided additional evidence for causality of *KRT5* mutations for this lethal form of EB. This mutation, which is previously unreported, affects the canonical splice site at the intron 7/exon 8 border.

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This variant was confirmed by bidirectional Sanger sequencing as homozygous in the proband (Fig. ~~2c~~2f). The parents, clinically unaffected, were heterozygous carriers of the corresponding mutation. The allele frequency of this mutation in 249,994 genomes in different populations was 0.

Consequences of the *KRT5* splicing variant at expression level

To further identify the effects of the *KRT5*: c.1440-1G>A variant on the level of mRNA expression and the splicing pattern of *KRT5*, whole-transcriptome profiling was performed by RNA-Seq with RNA isolated from the keratinocytes of the proband in comparison to six controls. Examination of the Sashimi plots revealed retention of intron 7 sequences in the mutant mRNA. Thus, the canonical splice site mutation in *KRT5* results in altered splicing predicted to cause aberrant mRNA and nonsense-mediated mRNA decay, as reflected by the reduced levels of the corresponding transcript, as visualized by heat-map analysis (Fig. 2g,h~~c~~). Western blotting of protein synthesized by keratinocytes established from the skin of the patient showed markedly reduced levels of keratin 5, when compared to an unaffected control (Fig. ~~2c~~2i). It should be noted that the polyclonal antibody used for Western blotting also reacts with keratin 6 which may explain the faint, but detectable, signal in the patient. The monoclonal antibody which yielded completely negative staining for keratin 5 in immunostaining (see below), does not work well in Western blotting.

Consequences of the *KRT5* mutations on skin ultrastructure

Histopathology of perilesional skin from the Proband 1 revealed separation of the epidermis from the underlying dermis: semi-thin sections located the blisters within the basal keratinocytes

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(Fig. 3a) which was confirmed by transmission electron microscopy (Fig. 3b). Adjacent to areas of blistering, aggregation of some keratin filaments was also noted (Fig. 3c), and the architecture of the intermediate filaments was perturbed with an unusual radial configuration of the keratin fibers (Fig. 3d). In the skin of the mother of the proband, some basal keratinocytes showed compacted basket-weave patterning, while in the heterozygous father's skin, the basal keratin filaments appeared condensed (Fig. 3e,f). Based on these observations, it was concluded that the proband has a form of EBS with basal keratin filament disruption, while the clinically unaffected parents demonstrated more subtle, slightly abnormal ultrastructural changes in the keratin intermediate filament network architecture within the basal keratinocytes.

Immunofluorescence of the proband's skin in Family 1 revealed expression of keratin 5 and keratin 14 both in the roof and the floor of a blister at the lower pole of the epidermis (Fig. 4a,d). In the epidermis of the proband's father, the keratin 5 and 14 distribution appeared normal, but the intensity of immunofluorescence was somewhat reduced compared to healthy, unrelated controls (Fig. 4b,c,e,f), however, no evidence of blistering was noted. Thus, these findings in the patient are consistent with an autosomal recessive form of EBS due to a homozygous missense mutation in keratin 5; the findings in the father are not specific beyond indicating slightly reduced immunoreactivity for keratins 5 and 14.

In Family 2, keratin 5 and keratin 14 staining in the KRT5:p.Asp158Asn homozygous individual revealed an irregular appearance of labeling (Figure 4). In Family 3, immunofluorescence staining for keratin 14 demonstrated normal levels of expression both in the roof and the floor of an epidermal blister. However, in contrast, staining for keratin 5 was entirely negative indicating absence of this keratin, fully in keeping with the proband being a human *KRT5* "knock-out" (Fig. 4).

Discussion

We have characterized three families with biallelic homozygous *KRT5* mutations with intra-epidermal blistering. In the proband of Family 3, the splicing mutation when homozygous resulted in complete absence of keratin 5, as determined by immunofluorescence staining, and this patient therefore can be considered to be a human "knock-out" of the *KRT5* gene. In Family 2, the proband was homozygous for a missense mutation, p.Asp158Asn, in *KRT5*, the clinically unaffected parents being heterozygous carriers of the mutation. Since the parents did not display any clinical findings related to EB, this missense mutation can be considered as a cause of an autosomal recessive form of EBS. The proband in Family 1 was homozygous for a *KRT5* missense mutation: p.Glu170Lys, and the parents were heterozygous carriers of this mutation. While the parents showed electron microscopic evidence of perturbed intermediate filament assembly in the basal keratinocytes, they did not show, under normal living conditions, clinical manifestations of EBS, although strenuous and prolonged walking was reported by them to result in mild blistering of the feet. Since this mutation, when heterozygous, resulted only in a subclinical phenotype, this mutation can also be considered to be recessive, manifesting as overt EBS only when present in both alleles.

Previously, homozygous or compound heterozygous *KRT14* mutations have been reported in an apparently autosomal recessive form of EBS [20-24]. In addition, we have recently provided evidence of semi-dominant inheritance in a family with a *KRT14* missense mutation [25]. In the latter family, the patients homozygous for the mutation demonstrated much more severe disease than the family members with monoallelic mutation. For *KRT5*, to the best of our knowledge, no patients with homozygous biallelic mutations have been reported.

However, Yasukawa *et al.* (2002) reported a family in which the proband was compound heterozygous for *KRT5* mutations p.Glu170Lys and p.Glu418Lys [26]. The first mutation, which is the same as discovered in our Family 1, was shown in their study to be associated with mild blistering phenotype when monoallelic and was considered to be autosomal dominant. In our study, the p.Glu170Lys mutation, when monoallelic, resulted in subclinical blistering and was designated as an autosomal recessive variant. The differences in the phenotypic expression of this mutation in the two families may reflect influence of modifier genes on the different ancestral background of these patients (Japanese *vs.* Iranian). The second mutation in the family reported by Yasukawa *et al.* (2002), p.Glu418Lys, was clearly recessive as the heterozygous carriers did not show any evidence of blistering. Thus, these authors interpreted their proband to display dominant and recessive compound heterozygous mutations in EBS. Similar combinations of dominant and recessive mutations in *COL7A1* and *COL17A1* genes have also been reported in families with DEB and JEB, respectively [27-29].

Among the 21 mutant genes associated with EB, several of them have been shown to harbor biallelic recessive null mutations, including our new demonstration of *KRT5*. Individually, these human "knock-outs" provide insight into the specific contributions these particular proteins provide physiologically to the prevention of blister formation in the skin, and they collectively underscore the molecular complexity in maintaining cell adhesion and integrity in human skin. The results also highlight the unmet medical needs for treatment of various forms of EB and propose novel personalized approaches for development of efficacious therapies for this group of disorders [30]

Materials and Methods

Patient data

This study was approved by the Institutional Review Board of the Pasteur Institute of Iran, and the Ethics Committee of the University of Freiburg. All subjects and the parents of children gave written informed consent to participate in research and to publish their image. A total of 512 families, including 362 from Iran, were subjected to mutational analysis. The criterion for study inclusion was a tentative diagnosis of epidermolysis bullosa (EB) based on history of neonatal cutaneous blistering suggestive of a genetic skin fragility disorder. Three patients were found to have a trauma-induced blistering due to homozygosity of *KRT5*.

Whole exome sequencing

DNA was extracted from peripheral blood lymphocytes of the proband by salting out method. DNA concentration was measured using a Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The qualified genomic DNA samples were randomly fragmented by Covaris, and the size of the library fragments was distributed predominantly between 150 and 200 base pairs (bp). Adapters were ligated to both ends of the resulting fragments. The adapter-ligated templates were purified by the Agencourt AMPure SPRI beads and fragments with insert size about 176 bp were excised. Extracted DNA was amplified by ligation-mediated PCR (LM-PCR), purified, and hybridized to the SureSelect Biotinylated RNA Library for enrichment. The hybridized fragments were bound to strepavidin beads whereas non-hybridized fragments were washed out after 24 h. Captured LM-PCR products were subjected to Agilent 2100 Bioanalyzer to estimate the magnitude of enrichment. Each captured library was then loaded on HiSeq2000 platform, and high-throughput sequencing for each captured library was performed to ensure that each sample met the desired average sequencing depth.

Raw image files were processed by Illumina Software 1.7 for base-calling with default parameters, and the sequences of each individual were generated as 90/100 bp pair-end reads. The bioinformatics analysis was applied to the sequencing data ("raw data") which was generated from the Illumina pipeline. First, the adapter sequence in the raw data was removed, and low-quality reads with too many uncertain nucleotide reads or low base quality were discarded. This step produced the "clean data". Secondly, Burrows-Wheeler Aligner (BWA) was used for alignment. Read groups were added, duplicates were marked, and the reads were sorted using Picard (http://broadinstitute.github.io/picard). After these processes, the final BAM files were used for variant calling using the GATK haplotype caller to identify both single nucleotide variants (SNVs) and indels. Candidate variants were then annotated using ANNOVAR software. ANNOVAR MetaSVM scores, based on several predictive bioinformatics tools, including SIFT, PolyPhen2, MutationTaster and MutationAssessor, were used to identify variants as deleterious.

Variant interpretation

Single nucleotide variants and copy number variants were examined using the annotated CSV files of WES from the proband. The sequence variants were filtered from the VCF files for missense, nonsense, and splice site-affecting variants. Indel variants were filtered for exonic in-frame insertions and deletions, frameshift mutations, and gained-lost start or stop codon. Additionally, only variants with 1000 Genomes database total frequencies of <0.001, or those without frequency data available, were examined. These variants were then overlapped with regions of homozygosity (ROH) in the patient. Considering the given phenotype, segregation analysis in the family, and the presence of several ROH in the patient with consanguineous parents, allowed us to identify a likely pathogenic homozygous variant in *KRT5* in Case 3.

Homozygosity mapping

For elucidation of ROH from the WES data, BAM file was converted to VCF format and used as an input for PLINK (http://pngu.mgh.harvard.edu/).

The Runs of Homozygosity algorithm in PLINK was used to identify ROH. The PLINK default values are appropriate for finding large segments of ROH present on dense genotyping platforms and were unchanged during the analysis. The PLINK output was filtered to eliminate ROH <4 Mb. (For technical details of homozygosity mapping) [31, 32].

Transcriptome profiling of keratin gene expression by heatmap analysis

Total RNA was extracted from the keratinocytes obtained from the Patient 3 and six healthy controls using TRIzol® Reagent and quantified on a Nanodrop ND-100 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), followed by RNA quality assessment on an Agilent 2200 TapeStation (Agilent Technologies, Palo Alto, CA). Multiplexed library construction, workflow analysis, and sequencing runs were performed following standard Illumina protocols (Illumina, Inc., San Diego, CA) using the TruSeq Stranded Total RNA kit with indexes from Set A (Cat #: RS-122-2301). Paired-end (2 × 75) sequencing reads were generated on the Illumina NextSeq 500 platform and stored in FASTQ format. FASTQ quality was checked using FastQC, and the TruSeq RNA adapter sequences were removed by Trimmomatic. Alignment and mapping were performed using STAR-2pass (v. 2.5.3a) [33] with the human reference genome (GRCh38/hg38) and GENCODE V27 annotations. In the first pass, an initial alignment was executed and the splice junction information was collected. This information was then used for the second pass in which the final alignment was performed. The alignments were sorted, read

group was added, and the corresponding index was created (using Picard tools). The static images of Sashimi plots were generated using the Integrative Genomic Viewer (IGV) [34]. R programming language (https://www.r-project.org/) was used to perform the expression analysis and plotting to show the distribution range of expression values of several keratins and representative housekeeping genes in patients *versus* six controls.

Western blotting

Cell lysates were collected from dermal keratinocyte cultures of patient 3 using RIPA lysis buffer (Sigma–Aldrich) supplemented with protease (Roche) and phosphatase (Sigma–Aldrich) inhibitors and subjected to SDS-PAGE on 10% Bis-Tris gel. Subsequently, proteins were transferred to a nitrocellulose membrane and immunolabeled with Anti-Cytokeratin 5 (ab53121) Rabbit Polyclonal antibody (1:500, ABCAM) and horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technologies, Danvers, MA, USA). Membranes were developed using the SuperSignal West Dura Extended Duration Substrate kit (Thermo Fisher Scientific, Life Technologies Europe, Ghent, Belgium) and scanned with the Amersham Imager 680 (GE Healthcare Life Sciences). Following stripping, the membrane was reprobed with an antibody to ERK (Abcam, Cambridge, United Kingdom) in order to check for equal loading. This analysis was repeated three times. Intensity of the bands was quantified using the ImageJ software.

Immunofluorescence staining

Skin sections (5 μm) were air-dried and initially blocked with diluted normal goat serum (Sigma-Aldrich, Dorset, UK), and then incubated with anti-KRT5/KRT14 antibody diluted 1:10 in

phosphate-buffered saline with 30% w/v bovine serum albumin (anti-cytokeratin 14 antibody, LL002, ABCAM; anti-cytokeratin 5 antibody, XM26, ABCAM; anti-cytokeratin 5 & 6 antibody, D5/D6 B4, ABCAM). After washing in phosphate-buffered saline, slides were labeled with fluorescein isothiocyanate secondary antibodies (Invitrogen, Paisley, UK). Negative controls omitting the primary antibody were also performed. All sections were photographed using the same camera and identical exposure times (three seconds).

Transmission electron microscopy

Skin biopsy specimens were cut into small pieces (<1 mm^3) and fixed in half-strength Karnovsky fixative for 4 h at room temperature. After washing in 0.1 M phosphate buffer (pH 7.4), the samples were immersed in 1.3% aqueous osmium tetroxide (TAAB Laboratories, Berkshire, UK) for 2 h, followed by incubation in 2% uranyl acetate (Bio-Rad, Hertfordshire, UK), dehydrated in a graded ethanol series, and then embedded in epoxy resin via propylene oxide (TAAB Laboratories). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

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Figure Legend

Figure 1. Clinical presentation of probands with homozygous biallelic *KRT5* mutations. (a) The Patient 1, a 32-year-old female, presented with widespread trauma-induced cutaneous blisters since birth. The blisters were particularly severe in the plantar aspects of the feet accompanied with plantar keratoderma. (b) The Patient 2, a male at the age of one year, characterized by generalized skin blistering since birth. (c) The Patient 3, a neonate with severe generalized EBS presented with fragile skin and mucosa. Milia was present at one week of life. The patient died shortly after birth.

Figure 2. Identification of biallelic *KRT5* mutations confirmed the diagnosis of autosomal recessive EBS. Pedigrees of the families 1 (a), family 2 (b), and family 3 (c), with probands indicated by arrowheads, revealed consanguinity. Sanger sequencing of the proband's DNA confirmed the presence of homozygous biallelic missense mutations in *KRT5* 21-gene panel. (d) WES identified 88,210 annotated variants in the Patient 3 which were filtered by steps indicated, resulting in identification of a homozygous mutation in *KRT5*. (f) Sanger sequencing confirmed the mutation. (e) Homozygosity mapping revealed that the *KRT5* gene resides within a 22.3 Mb ROH (blue blocks) in chromosome 12. (g) Heatmap visualization of transcriptome analysis revealed markedly reduced level of *KRT5* expression in comparison to other keratin genes, other genes associated with EB phenotypes and randomly selected housekeeping genes.

(h) Sashimi plot of the transcriptome profile of the mutant *KRT5* mRNA revealed complex splicing, including retention of intron 7 (asterisk) and intron 8 sequences. (i)

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Western blot revealed markedly reduced levels of keratin 5 in keratinocytes isolated from the skin of Patient 3

Figure 3: Skin electron microscopy reveals low level intra-epidermal cleavage and keratin filament abnormalities. (a) Semithin sections of skin from Patient 1 with homozygous *KRT5* mutation reveal blister formation (asterisk) close to the dermal-epidermal junction; (b) Ultrastructurally, the plane of cleavage (asterisk) is within the lower pole of basal keratinocytes, with fragments of basal cells present in the blister base (arrow); (c) Within basal keratinocytes there is blister formation (asterisk) and aggregation of some keratin filaments (arrow); (d) In some basal keratinocytes, the architecture of the intermediate filaments is perturbed (arrows), with a more radial configuration of the keratin rather than a concentric perinuclear configuration; (e) In the heterozygous mother's skin, some basal keratinocytes show a compacted basket-weave pattern (arrow); (f) In the heterozygous father's skin the basal keratin filaments appear condensed (arrow) in some basal keratinocytes.

Figure 4: Immunofluorescence microscopy reveals alterations in basal keratin labeling patterns. Upper panel (a) Keratin 5 staining in patient 1 with the homozygous *KRT5*:p.Glu170Lys mutation reveals an irregular, slightly punctate appearance of labeling within the lower epidermis as well keratin 5 mapping to the base and roof of blister (asterisks); (b) In the heterozygous father, there is also some irregularity and unevenness to the keratin 5 staining in the lower epidermis with slightly reduced overall immunoreactivity; (c) Control skin labeling for keratin 5 reveals even intracellular staining in the lower epidermis particularly in basal keratinocytes; (d) Keratin 14 staining in the homozygous individual with *KRT5* mutation reveals

an irregular appearance to labeling within the lower epidermis as well keratin 14 mapping to the base and roof of a blister (asterisks); (e) In the heterozygous father, there is also some irregularity and unevenness to the keratin 14 staining in the lower epidermis and slightly reduced immunoreactivity; (f) Control skin labeling for keratin 14 reveals even intracellular staining in the lower epidermis particularly in basal keratinocytes. (g and h) Keratin 5 and keratin 14 staining in the homozygous *KRT5*:p.Asp158Asn proband (patient 2) reveals an irregular appearance to labelling. (i and j). In the homozygous *KRT5*: c.1440-1G>A proband (patient 3), keratin 5 is essentially absent, and keratin 14 staining shows cleavage within the basal keratinocytes. (k and l) The panels show control skin labeling for keratin 5 and 14 with even intracellular staining in the lower epidermis, particularly in basal keratinocytes. Scale bar = 50 µm.